Supplement Figure S1

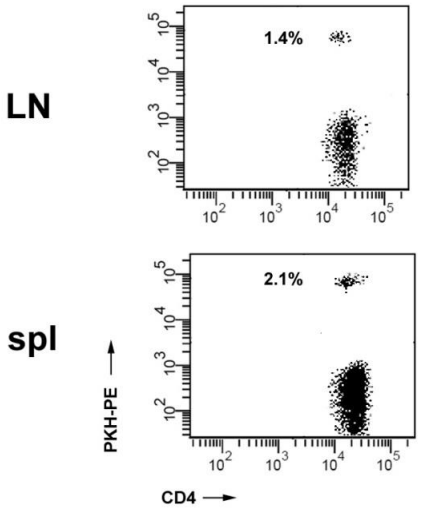

Fig. S1: Injected Treg home to lymph nodes (LN) and spleens (spl).

Treg were isolated, stained with the fluorescent dye PKH-PE and $3x10^6$ cells/mouse were i.v. injected. 24h later single cell suspensions were prepared from spl and cervical LN and stained for CD4 (FITC). FACS analysis revealed PKH-PE^+ Treg in LN and spl. The numbers indicate the percentage of injected Treg among $CD4^+$ T cells.

Supplement Figure S2

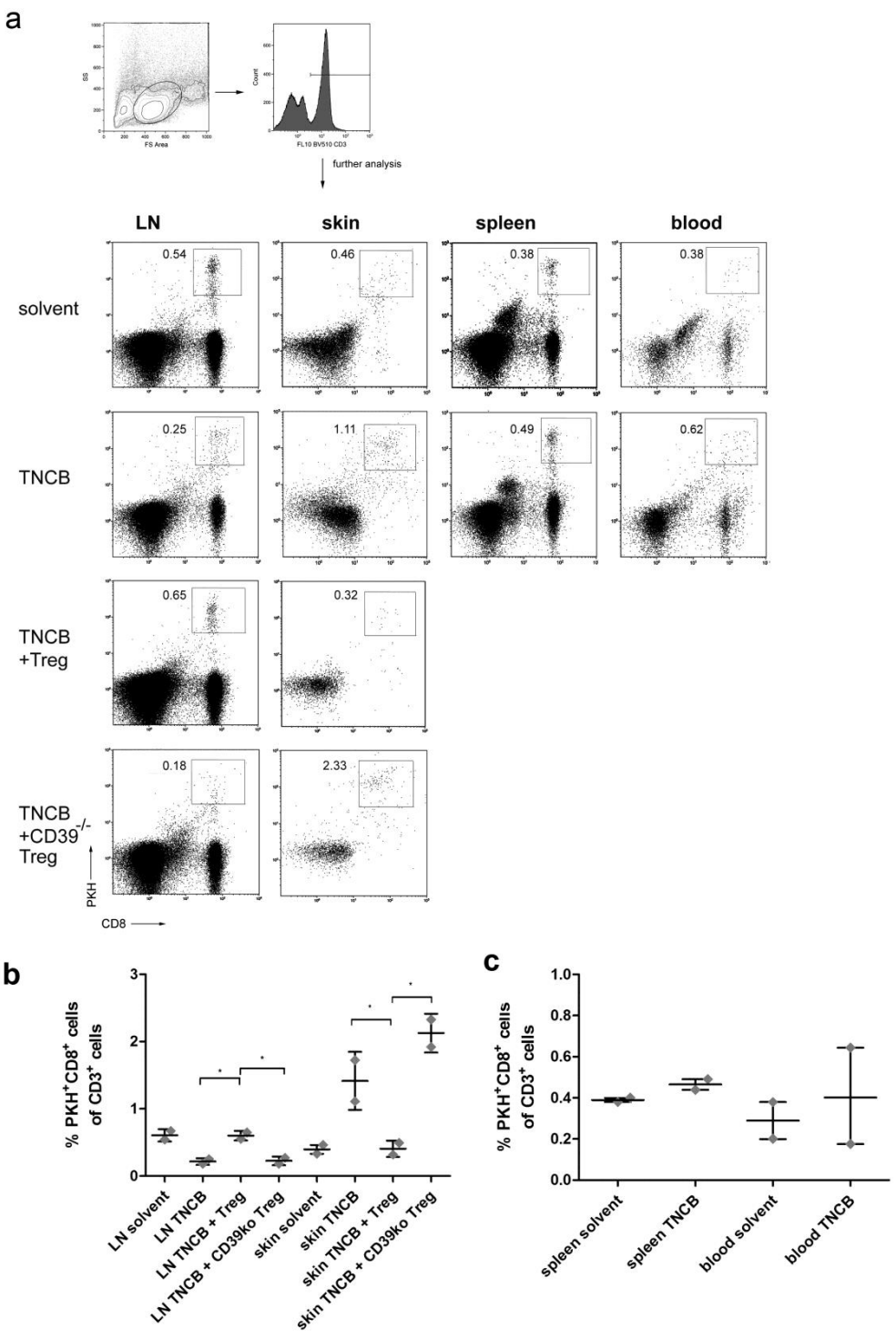

Fig. S2: Effect of Treg on distribution of PKH-PE labeled CD8$^+$ T cells in different organs after sensitization. Isolated CD8$^+$ T cells were labeled with PKH-PE and i.v. injected into animals (3x10^6 cells/mouse). 24h later Treg were i.v. injected as indicated. Some groups were left untreated. After further 24h, mice were sensitized with TNCB or treated with solvent at the ear and sacrificed 2h later. LN cell were stained for CD3 and CD8 and analyzed by flow cytometry. **(a)** shows a typical analysis, numbers indicate the frequency of CD8$^+$PKH-PE$^+$ T cells among CD3$^+$ T cells. As in blood and spleen no difference between solvent and TNCB groups were apparent, injection of Treg was omitted. **(b)** and **(c)** shows the mean ±SD of 2 independent experiments. * indicated a significant ($p<0.05$; t-test) difference.

Supplement Figure S3

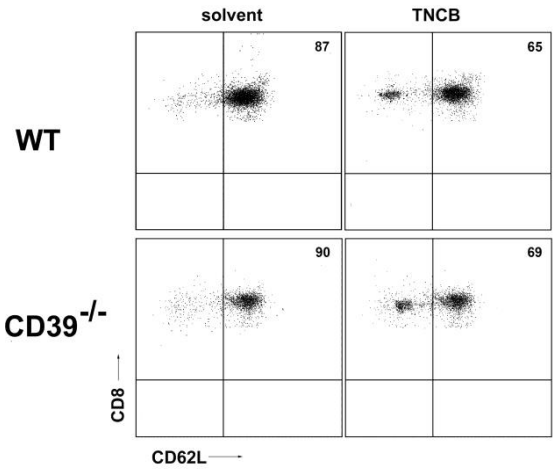

Fig. S3: No difference in CD62L expression by LN CD8$^+$ T cells in CD39$^{-/-}$ and wt mice after TNCB sensitization.

Wildtype (WT) and CD39$^{-/-}$ mice were sensitized with TNCB in acetone/olive oil or treated with solvent only at the ears. 2h later draining LN were removed. Single cell suspensions were stained for CD8 and CD62L and analyzed by FACS. Gated CD8$^+$ T cells are shown in a dot plot from a typical experiment (out of 3 experiments). Numbers indicate percentage of cells in quadrant.

Supplement Material and Methods S4

Mice and reagents

Wildtype C57BL/6N mice were purchased from Janvier (Janvier Labs, Saint Berthevin, France). CD39 deficient ($CD39^{-/-}$) mice were backcrossed onto C57BL/6N mice for 7 generations and housed under SPF conditions in the central animal facility of the University of Heidelberg. Female mice between 7 and 9 weeks of age were selected for the experiments. All experiments were approved by the state of Baden Württemberg.

Complete medium (CM) was RPMI supplemented with 10% FCS, Pen/Strep, HEPES and Glutamine, all purchased from GE-Healthcare, Freiburg, Germany. Standard chemicals were purchased (if not stated otherwise) from Carl Roth, Karlsruhe, Germany. The P_2X_7 antagonist KN-62 was from Tocris (Bio-Techne, Wiesbaden, Germany) and was used at a final dilution of 10µM, according to Ring et al. (Ring et al. 2011). The ADAM17 antagonist TAPI-1 (10µM final dilution) was from EMD (Merk-Millipore, Schwalbach, Germany). Substances were dissolved in DMSO and used in cell cultures at least in final dilutions of 1:1000. Controls contained equal amounts of the solvent. The purified ATPase was purchased from Roche (Mannheim, Germany) and used in a concentration of 2U, according to Yip et al. (Yip et al. 2009).

Induction of CHS

Mice were sensitized by painting 15µl of 1% 2,4,6-trinitro-1-chlorobenzene (TNCB; Sigma-Aldrich, Germany) dissolved in acetone/olive oil (4:1) or 15µl of solvent only on the shaved abdomen. In some cases ears were used for sensitization (as indicated in the figure legends), that way the respective draining lymph nodes were clearly defined.

Preparation of single cell suspensions, purification of T cell subsets and labeling

For single cell suspensions, skin draining lymph nodes were minced and strained through a 70 µm cell strainer (Falcon, Becton Dickinson Heidelberg).

$CD4^+CD25^+$ T cells (Treg) as well as $CD4^+$ and $CD8^+$ T cells were prepared from single cell suspension of spleens and LN using commercially available magnetic bead enrichment kits ($CD8a^+$- or $CD4^+$-T cell isolation kits and $CD4^+CD25^+$ Regulatory-T cell isolation kit; Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The purity of the isolated cell populations was always confirmed by flow cytometry.

For cell labeling with PKH26 (PKH-PE; Sigma-Aldrich, Taufkirchen, Germany), $2x10^7$ freshly isolated serum free cells were resuspended in 1 ml diluent C before adding 1 ml of freshly prepared PKH26 (4µM). After 3min incubation at room temperature, the reaction was stopped by adding 2ml FCS for 1min. Thereafter 4ml medium was added and the labeled cells were collected by centrifugation (400xg, 10min, 25°C). If not stated differently $3x10^6$ cells/mouse (Treg, $CD8^+$ T cells) were injected in 100µl PBS intravenously into the tail vein (Ring et al. 2010a; Ring et al. 2010b).

For analysis of PKH-PE^+ cells in blood, whole blood was collected in heparinized tubes and PBMCs were prepared via Biocoll separating solution (Biochrom AG, Merk-Millipore) centrifugation. Single cell suspensions were prepared from spleens by a collagenase digest (Collagenase IV, 800U/ml; Cell Systems, Germany) followed by straining through a 70µm cell strainer, erythrocytes were lysed (ACK buffer, Thermo-Fischer, Dreieich, Germany). For skin preparations ears were separated into dorsal and ventral halves with forceps and incubated in 0.5% Trypsin/PBS/5mM EDTA for 30min, 37°C, 5% CO_2). Reaction was stopped with 20% FCS/PBS/0.1% DNase. Ears were cut into small pieces and after vigorous shaking suspension were collected through a 70µm cell strainer. Amount of PKH-PE^+ cell

populations was analyzed by flow cytometry (FACS-Canto; BD, Heidelberg, Germany or Gallios Cytometer, Beckman-Coulter, Krefeld, Germany).

Flow cytometry and functional assays

Cell suspensions were adjusted to $1x10^6$ cells/ml in PBS/3% FCS (v/v), 500µl aliquots of cells were stained (as indicated) with 1µl of the following antibodies: CD4-FITC (equals 1µg/ml), CD8-APC (equals 0.4µg/ml), CD62L-PE (equals 0.4µg/ml), CD69-PE (equals 0.4µg/ml) (all from e-Bioscience, Frankfurt, Germany) and CD49e-PE (equals 0.4µg/ml) (Biolegend, Fell, Germany) and analyzed by flow cytometry (FACS-Canto; BD, Heidelberg, Germany and Gallios Cytometer, Beckman-Coulter, Krefeld, Germany). For in vitro experiments LN cells were cultivated in CM in 24 well plates (Falcon, BD, Heidelberg, Germany) with graded doses of ATP (Sigma, Deisenhofen, Germany) for indicated times and analyzed by FACS (see above).

For detection of ATP, draining LN were removed from sensitized mice and individually placed in 100µl medium into single wells of a 96 well plate. Under microscopic control LN were disrupted by tweezers and the supernatant was immediately centrifuged and assayed for ATP. For restimulation of the LN cells, isolated LN cells were incubated for 2h with 10µM TNBS (2,4,6-trinitrobenzenesulfonic acid solution, Sigma Aldrich, Germany). Amount of ATP was determined in aliquots of tissue culture supernatants in the MicroBeta® TriLux from PerkinElmer using a commercially available assay (ATPlite, Perkin Elmer).

For DC-T cell cocultures DC were prepared as previously described (Ring et al., 2010b). Briefly, bone marrow cells were grown in CM supplemented with 10ng/ml rmGM-CSF and 10ng/ml rmIL-4 (Miltenyi) with intermittend washings. If required, DC were haptenized with 10mM 2,4,6-trinitro-benzenesulfonic acid (TNBS, Sigma-Aldrich) for 10min at 37°C. The reaction was stopped by adding FCS for 1 min, followed by several washings with CM.

Cocultures of $1x10^6$ T cells and $1x10^5$ DC or $5x10^4$ DC, respectively, were set up in 1ml CM in 24-well plates (Falcon) and tissue culture supernatant was assessed for IL-10 and IFNγ. Cytokine levels were determined by sandwich enzyme linked immune-sorbent-assay (ELISA), using paired antibodies (ELISA Ready-SET-Go, eBioscience).

Western blotting

Cell suspensions were lysed in PBS/1% Triton X-100/protease inhibitor cocktail (Roche, Mannheim, Germany). Protein content was determined by BCA (Thermofischer, Dreieich, Germany) and 10µg protein was loaded on 7.5% standard PAA gels and transferred onto PVDF membranes (all reagents from Peqlab, Erlangen, Germany). ADAM17 (Santa Cruz Biotech, Heidelberg, Germany) antibodies were diluted 1:500 and ERK/pERK antibodies (Cell Signaling, Frankfurt, Germany) were used at 1:600. Staining with anti-Actin (1:500; Santa Cruz Biotechnology, Heidelberg, Germany) served as loading control. Blots were visualized by HRP-labeled secondary reagents (Dianova, Hamburg, Germany) and chemiluminescence (ECL; GE-Healthcare, Freiburg, Germany). To detect shedding of CD62L, isolated cells were incubated with ATP for 3h in CM and 2 ml aliquots of the tissue culture supernatants were subjected to over-night immunoprecipitation with 5µl anti-CD62L (clone LAM-11). Soluble CD62L was precipitated using Protein A/G Sepharose (Santa Cruz Biotech, Heidelberg, Germany) and analyzed by polyacrylamide gels and silver staining (Thermo Fisher Scientific, Waltham, MA USA).

Statistics

As indicated in the figure legends t-test or ANOVA (for 3 and more groups) was used to test for significance using GraphPad Prism software (Statcon, Witzenhausen, Germany).